LIVER NUCLEAR HISTONES OF ZINC-DEFICIENT RATS

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By

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A C K N O W L E D G M E N T

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LIST OF ABBREVIATIONS

C.M. Cellulose	- Carboxymethyl cellulose
DFP	- Diisopropylfluorophosphate
DNP	- deoxyribonucleoprotein
DNA	- deoxyribonucleic acid
EDTA	- ethylenediaminetetraäcetic acid
EIA	- ethyl iodoacetate
RNA	- ribonucleic acid
m-RNA	- messenger ribonucleic acid
ma	- milli amperes
p.p.a.	- parts per million
r.p.m.	- revolutions per minute
x g	- times gravity

CHAPTER I

INTRODUCTION

A. Zinc Deficiency

In 1869 Raulin (1) recognized zinc as an essential element. Since then, a great deal of evidence substantiating the universal importance of this micronutrient has accumulated. States of zinc deficiency are described in *Aspergillus niger* (1), *Neurospora crassa* (2-4), corn (5), tomato (6), and other plants. In the animal kingdom, indisputable evidence for the specific effects of zinc deprivation in the rat was presented by Todd *et al.* (7), and the Wisconsin group (8-10). Subsequently, zinc deficiency syndromes were documented for swine (11), chicken (12, turkey (13), calves (14), lambs (15), and the Japanese quail (16).

Despite the overwhelming evidence for the existence of zinc deficiency states, the nature of metabolic disturbances it causes remains unknown. The subcellular distribution of zinc in the rat liver follows closely the intracellular localization of known zinc metalloenzymes (17). These have been shown to lose activity when their zinc is bound with complexing agents, and to regain activity after replacement of the zinc (18, 19).

Hence, it may be expected that zinc deprivation should result in loss or reduction of activity of most zinc metalloenzymes. In fact, alcohol dehydrogenase was absent in zinc-deficient mycelia of Neurospora crassa (3-4). Subnormal levels of intestinal and kidney alkaline phosphatase activities in the rat were reported (10, 20-21). Also, bone alkaline phosphatase activity was lowered in zinc deficient turkeys (22). However, carbonic anhydrase, and lactic dehydrogenase, both zinc dependent enzymes, were found to be unaffected by zinc deficiency in rats (23), and chicken (12). Furthermore, in the male genital tract which accumulates high concentrations of zinc, the amounts of the metal do not reflect the activities of known zinc metalloenzymes (24).

Impaired growth in plants and animals during zinc deficiency has indicated that protein synthesis is one of the biochemical loci affected. Changes have been found in the levels of plasma proteins of zinc-deficient rats (9), chicks (25), and the Japanese quail (26, 27). Lower protein content in zinc-deficient mycelia of Neurospora crassa (3, 4), and tomato (6) has also been reported.

Zinc has been suggested to have a role in protein, m-RNA and DNA syntheses. Schneider and Price (28) have hypothesized that decreased levels of RNA may be the cause of growth inhibition during zinc deprivation. In fact,

investigations of the effect of zinc deficiency upon RNA, and protein synthesis, in Euglena gracillis (29), Rhizopus nigricans (30), and Mycobacterium smegmatis (31, 32) have described a general pattern. The metabolic disturbances associated with zinc deficiency, they believe, starts with the arrest of RNA formation; soon protein synthesis stops. Later, the rising total nitrogen and DNA levels associated with growth come to a standstill. Addition of zinc to the culture media stimulates RNA and protein synthesis (30). Further, it is of interest that in experiments performed by Fox and Harrison (27), Actinomycin D, the inhibitor of DNA primed m-RNA formation, influenced zinc-deficient birds only, during short periods of fasting. This implies disturbed m-RNA formation during zinc deficiency. However, injection of growth hormone, which stimulates m-RNA formation, caused retention of nitrogen but no weight gain in deficient rats, a finding that does not support direct m-RNA involvement.

Nuclear basic proteins, the histones are known to be involved in DNA dependent RNA synthesis. As an example, histones in 100 µg/ml concentration activated de novo synthesis of lactic dehydrogenase, a zinc metalloenzyme from embryonic chick brain in tissue culture. While, at 400 µg/ml complete inhibition of the enzyme synthesis occurred (33). Since histones could inhibit the formation

of a zinc-dependent enzyme *in vitro*, and probably other proteins *in vivo*, the possibility that during zinc deficiency synthesis of some key metalloenzymes were blocked by histones needs to be considered. If histones behaved as repressors, definite changes in histone concentration or type might be expected during zinc deprivation. For these reasons a study of histone behavior was made in zinc-deficient rats and compared with that of controls receiving appropriate quantities of zinc.

B. Histones

The histones, basic proteins of molecular weight 8000-130000, are found in close association with the DNA of the nucleus of somatic cells (34). Their metabolic role is not known. However, keen interest was aroused in them by Stedman *et al* (35) who suggested a genetic regulatory role for them. Significant findings concerning heterogeneity, species and organ specificity and nature of interaction of histones with the DNA have been described (36).

The heterogeneity of histones is well established. Differential extraction and precipitation, ion exchange chromatography, gel filtration, electrophoresis (37,38), and countercurrent distribution (39) techniques have been used to separate histones into three main fractions:

arginine-rich (F_1), moderately lysine-rich (F_2), and lysine-rich (F_3). Each has been further subfractionated. Starch gel electrophoresis (40, 41) of whole, as well as chromatographically separated fractions (42, 43) of histones yields multiple bands. Extensive heterogeneity was revealed by the use of polyacrylamide gels (40, 44). Moreover, Murray (45) demonstrated that histones prepared by these widely different methods exhibited heterogeneity. The present belief is that there are a limited number of histones which have remarkable similarities in electrophoretic patterns (40, 46), amino acid compositions (47, 48) and NH_2-terminal amino acids (49, 50). Comparisons of these characteristics were made with calf thymus histones which have been most completely studied.

Species and organ specificity of histones have been investigated. Neelin (40, 51) and Hnilica (42) isolated a unique histone from chicken erythrocytes. Differences from various organs of chicken (40) and rats (42) have also been described, as have slight differences between histones of the same organs of the rat, guinea pig, and the rabbit (52). However, histones from mouse brain, and liver (53), calf thymus, liver and kidney (50), and rat brain, liver and kidney (42) showed no detectable differences.

An interesting succession of histone types during spermatogenesis in the squid Loligo (54) and fertilization in the snail Helix aspera (55) were reported. Concomitantly, newborn and adult tissues from chicken showed minor differences (46). Also, slight but consistent differences in the histones of young rat liver, and brain were detected (52). Microorganisms grown under different conditions were investigated and significant differences in the histones were observed (56). Small differences between normal and neoplastic tissues have been also described (34).

C. Histone Metabolism

The site of biosynthesis of histones is unknown. The nucleus has been suggested (54, 57) although during spermatogenesis in the grasshopper, protein-bound labeled arginine appeared first in the cytoplasm of the sperm nucleus. Originally it was believed that histone and DNA syntheses occurred simultaneously (58, 59). Recent evidence indicates that histone synthesis is an independent process, and may take place before DNA synthesis (60, 61). Actinomycin D, the known m-RNA inhibitor, causes a decrease in the incorporation of labeled amino acids into histones (62). Moreover, inhibition of histone synthesis by puromycin indicates that histone biosynthesis is like that of other proteins (63, 64).

Histones undergo a slow but definite turnover (65, 53), although in avian erythrocytes and reticulocytes no turnover could be detected (66). C^{14} lysine is incorporated into the peptide structure of lysine-rich histone fractions (67). Furthermore, labeled amino acids are incorporated at different rates into histone fractions isolated from calf thymus nuclei (62, 64).

Methylation and acetylation of histones, which modify histone-DNA interaction, can occur both *in vivo* and *in vitro* (68, 62). These processes are not inhibited by puromycin (63). Recent evidence indicates that during gene activation in human lymphocytes, acetylation of histones (69) takes place just before the rise in RNA levels. Phosphorylation of histones also occurs (70). Serine phosphate has been identified as the site (71). Thiol groups have also been reported in histones (72).

The concentration of histones in the nucleus was assumed to be equivalent to that of DNA (73). However, Umãna *et al* have shown that in nondividing stable cells, histone concentration is twice that of DNA. Starvation lowers the histone:DNA ratio to 1.65. In tumors the ratio is about one (74). The amount of histones to DNA in adult tissues varies (75).

Basic groups of histones interact with phosphate groups of DNA primarily through nonspecific ionic linkages

(76, 77). The ease of dissociation of nucleohistone complexes by acidic or high ionic strength solutions has been presented as evidence (77). The results of analyses of tryptic finger prints indicate that basic amino acids in histones are not found in a regular order as are the phosphate groups of DNA (45, 78). Furthermore, the interaction of histones with DNA seems to be size and structure-dependent (79). Nucleohistone association and dissociation studies (80, 81) have shown that a lysine-rich histone fraction specifically associates with a DNA moiety rich in guanine and cytosine.

D. Histones in the Biochemistry of the Nucleus

Histones were shown to be involved in several reactions taking place in the nucleus. Among these are: synthesis of DNA-dependent RNA, of DNA and of nuclear ATP.

In isolated thymus nuclei, addition of histones inhibits DNA-dependent RNA synthesis (82, 83). While, enzymatic removal of histones with trypsin or acetylation, enhances it. Pea embryo native chromatin or reconstituted nucleoprotein (histone:DNA, 2:1) is inactive in supporting DNA-dependent RNA synthesis (73, 84). Selective removal of histones from reconstituted nucleoprotein stimulates the incorporation of labeled RNA precursors.

The degree of inhibition caused by different histone fractions is unknown (73, 82, 84). Furthermore, the concentration of histones in relation to DNA may be significant (33, 85).

The interaction of histones with DNA (86) and nuclear ATP (87) syntheses are less well understood. However, histones inhibit both of the above mentioned reactions. Hence, it is expected that most phosphorylating and energy requiring processes in the nucleus may be adversely affected.

CHAPTER II

MATERIALS AND METHODS

A. Induction of Zinc Deficiency in Rats

Weanling rats, all male, of a local Sprague-Dawley strain were sorted at random into experimental and control groups. The rats were housed in stainless steel cages, and were pair-fed the appropriate diets after a twenty-four hours fast. Deionized water was provided *ad libitum*. The diet was based on that of Forbes and Yohé (88) with the following modifications: the protein was changed to 80 % casein (EDTA-purified as described in (89)) and 20 % gelatin. Cellulose and chlortetracycline were omitted. Instead of glucose, sucrose and dextrin served as carbohydrate sources. 1500 I.U. Vitamin D, and 12000 I.U. Vitamin A, and 1.0 g Tocopherol were added per 4.5 kg batch. The $CaHPO_4$ used in the salt mixture was prepared from Na_2HPO_4 and $CaCl_2$ by isoelectric precipitation to decrease zinc contamination found in commercially available $CaHPO_4$.

The zinc content of the food was measured with the aid of dithizone (90) or atomic absorption spectrophotometry (91), after digestion with sulfuric, nitric and perchloric acids (89). The zinc-depleted diet contained

less than two p.p.m. zinc. The controls received the same diet except that $ZnCO_3$ was added to raise the zinc level to 20 p.p.m.

The zinc content of hair was measured as a supplementary method for assessing zinc deficiency (89). Hair was clipped from the abdomen, washed, digested and analyzed by atomic absorption spectrophotometry (89).

The rats were weighed twice each week. As soon as severe zinc deficiency symptoms developed the experiment was terminated. This occurred twenty to forty days after starting diets.

B. Preparation of Rat Liver Nuclei

Rats fasted for twenty-four hours were anesthetized with ether, the abdominal and thoracic cavities were opened. Blood was withdrawn with a syringe from the left ventricle. A catheter was introduced into the vena cava and the liver was perfused *in situ* with ice-cold 0.14 M NaCl to remove blood. Perfusion was continued until the liver became gray. All subsequent operations were carried out at 5° C or below.

The liver was rinsed with saline, blotted with filter paper, and one gram portions were minced with scissors and homogenized in twenty volumes of 0.25 M sucrose, 3 mM $CaCl_2$. Either 1 mM diisopropyl fluorophosphate

(DFP) or 0.1 mM ethyl iodoacetate (EIA) were added just before homogenization. DFP, and EIA were used to inhibit enzymatic hydrolysis of histones known to occur in the nucleus upon homogenization of the rat liver. A close fitting teflon pestle with serrated edge, and a Potter Elvehjem glass homogenizer were used. After numerous trials 6-8 strokes, 40 seconds and 3500 r.p.m. for the rotor were found to yield maximal number of intact nuclei. The homogenate was passed through four layers of cheese cloth, and centrifuged for ten minutes at 1000 x g. The crude nuclear sediment was resuspended in the homogenizing solution (1 g/10 ml) and was centrifuged as above. The pellet obtained was suspended as described by Chauveau (92) in 2.1 M sucrose using a loosely fitting teflon pestle with the glass homogenizer. The nuclear suspension corresponded to 5 g of the original tissue per 100 ml of 2.1 M sucrose solution. Heads 21 or 30 of the model L Spinco preparative ultracentrifuge were used for 75 minutes at 50000 x g calculated for the bottom of the centrifuge tube. Samples of the nuclei prepared were stained by hematoxylin hydrochloride and examined with the light microscope. The nuclear preparations contained little detectable contamination (whole cell or debris). Yields were low.

C. Preparation of Histones by Isoelectric Precipitation

Initially a procedure based upon isoelectric precipitation of histones was tested. Two histone fractions from the rat liver were obtained by a modification of Daly and Mirsky method (93). The nuclei were extracted with 0.25 N HCl and the clear supernatant solution obtained after centrifugation was titrated to pH 8 with 1 N NaOH. Turbidity, indicating aggregation, appeared first at pH 6 and at about pH 8 a cloudy, white precipitate formed. No further precipitation was observed upon raising the pH. After the first histone fraction was collected, the pH of the supernatant solution was raised to 10.0 and three volumes of 98 % ethanol were added. A white precipitate, the second histone fraction, was formed. The isoelectric precipitation procedure was abandoned because both histone fractions obtained were contaminated by other proteins having isoelectric points in the same pH range. Also, alkaline treatment of histones caused denaturation, hence loss of solubility.

D. Preparation of Deoxyribonucleoprotein (DNP)

A second procedure applied to isolation of histones depended upon separation of deoxyribonucleoprotein. All steps described previously in the high speed differential centrifugation of nuclei were followed. The nuclear

pellet was extracted with 2 M NaCl (50) for 5 hours. The supernatant solution was decanted and centrifuged 2 hours at 2000 x g. The new supernatant solution containing the DNP was diluted to a concentration of 0.15 M NaCl. The DNP precipitate was collected by centrifugation.

E. Purification of Histones

The clean nuclei or DNP were extracted with 0.25 N HCl for two to three hours. The extract was centrifuged and the clear supernatant solution containing the histones was purified by the method of Lindh and Brantmark (38).

The acid extract was treated with a saturated solution of freshly prepared ammonium Reineckate until complete precipitation occurred. The histone Reineckate was washed with an appropriate volume of 0.05 M Tris HCl pH 8.4 buffer. An equal volume of saturated Reineckate was added, and the precipitate was collected by centrifugation. The supernatant discarded contained proteins with isoelectric points below pH 7, together with low molecular weight basic contaminants. This step was repeated twice. The precipitate was treated with acetone HCl (98 parts acetone : 2 parts concentrated HCl) which had been cooled below -5° C, and centrifuged at -20°C. The precipitate containing the histones was extracted with β-alanine-acetate buffer at pH 4.5, or acetate buffer at pH 4.2.

An insoluble greenish precipitate remained. The flowsheet summarizes the different steps (Fig. 1).

F. Disc Electrophoresis of Histones in Acrylamide Gels

Disc electrophoresis was performed according to Reisfeld (94) in 7.0 % acrylamide gels. A combination of large and small pore gels at pH 6.7 and pH 4.3 respectively was used to produce the molecular sieving and electrophoretic effects required to resolve mixtures of closely related proteins (95, 96). Unless otherwise specified, a 6 ma constant current was applied for 65 minutes per tube. 7.5 cm long and 0.5 cm internal diameter glass running tubes were used. The gel stacked at pH 5.0 and ran at pH 4.3. The buffer used for electrophoresis was 0.35 M β-alanine-acetate pH 4.5. Under these conditions histones showed excellent resolution.

A volume of solution containing fifty microgram histone samples was applied. Larger protein samples are known to cause aggregation with loss of resolution; furthermore, scanning of thick bands is inaccurate. Protein concentration was measured by the method of Lowry et al (97). A preparation of calf thymus histone* was used for the protein standards.

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*HLY 61 A Worthington Biochemicals.

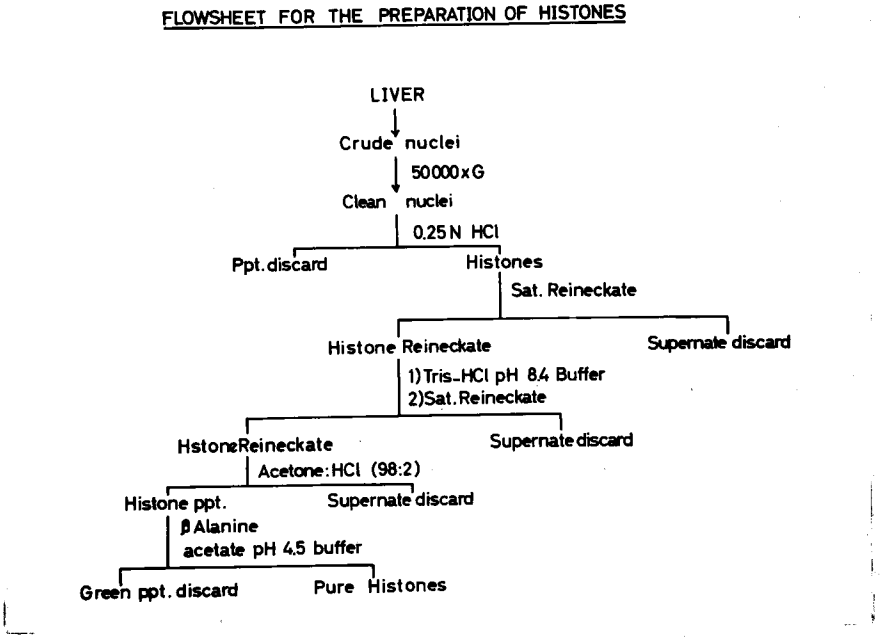

FIGURE I. FLOWSHEET FOR RAT LIVER NUCLEAR PREPARATIONS.

The histone sample was mixed with an equal volume of gel solution composed of 1 part B : 2 parts D : and 1 part E, where B, D and E are (94):

B. 48 ml 1 N KOH, 2.87 ml glacial acetic acid, 0.46 ml of N,N,N',N'-tetramethylethylenediamine in 100 ml water solution.

D. 10 g acrylamide, 2.5 g N,N'-methylenebis-acrylamide in 100 ml water solution.

E. 4.0 mg riboflavin in 100 ml water solution.

Addition of four parts water was omitted from the gel solution because the samples were dilute. Histones applied were either in water or in β-alanine-acetate buffer pH 4.5 or in acetate buffer pH 4.2. After the run, the gels were removed from the tubes with the help of a needle, and a syringe filled with 7 % acetic acid. While a gentle stream of the solution was released, the needle was inserted between the gel and the tube wall, and a complete turn around the gel made.

The gels were immersed in 0.5 % Amido Schwarz 10 B, in 7 % acetic acid, for a minimum of two hours. They were destained either electrophoretically in 7 % acetic acid with currents less than 8 ma per tube, or by diffusion in the same solvent. The latter was preferred because currents as low as 4 ma/tube removed fainter bands. Also development of artifacts is reported (98).

The destained gels were scanned with Aminco Fluoromicrophotometer equipped with a scanner running at a speed of one centimeter/minute. The photometer was connected to a Moseley model 680 recorder, and the gel patterns were traced. A wratten ND filter 1-10 % and a green filter 58 were used to decrease the intensity of light, and make the use of slit adjustment possible. An unstained section of the gel was selected to adjust the zero absorbance above the baseline. Several recordings were made at different slit widths. The tracing showing the best resolution of bands with the least noise was selected for analysis. Some variation in the optical properties of the gel which could not be avoided gave rise to minor differences in the optical density for the clear areas. Hence, the region of the more prominent bands (4 major, 2 minor) was analyzed only. The curves corresponding to the absorbance of the protein bands on the gel were assumed to have Gaussian symmetry. When two curves overlapped because two close bands could not be completely resolved by the scanner, the missing arms of the two curves were drawn symmetrically with the other. The area falling under the curves was measured with a planimeter. The average of five such measurements was recorded. Each peak was expressed as % of total area. Since the quantity of protein applied to the gel columns

was the same, the several preparations examined could be compared directly by areas under the curves.

G. Starch Gel Electrophoresis

Starch gel electrophoresis in acetate buffers of differing ionic strength and pH was also tested. The best results were obtained with acetate buffer pH 4.8, 0.02 ionic strength. Because at least 500 µg samples were required and resolution as compared to disc electrophoresis was poor, it was discontinued.

H. Paper Electrophoresis

The presence of nonbasic protein contaminants in substantial amounts was excluded by electrophoresis carried out on paper in veronal buffer pH 8.6, ionic strength 0.075 and a current of 2.5 ma/cell for 16 hours. The paper strips were stained with 1 % Amido Schwarz in 7 % acetic acid, and destained in the same solvent.

I. Fractionation of Histones by Carboxymethyl Cellulose

Rat liver histones were fractionated on C.M. Cellulose* columns according to the method of Johnes et al (99). The protein was added to the column in 0.1 M

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*C.M. Cellulose Sigma Chemical Co. medium mesh 0.60 mEq/g.

acetate pH 4.2 buffer. Three fractions were eluted by the use of 0.2 M acetate - 0.42 M NaCl buffer pH 4.2, 0.01 N HCl, 0.02 N HCl respectively. 20 x 1 cm columns were used. The C.M. Cellulose was previously washed with 4.2 pH acetate buffer to remove any ultraviolet absorbing moieties. In order to compare histones from zinc deficient and control rats, identical amounts of samples were added and eluted. Fractionation was carried out initially at room temperature. Later, experiments were performed at 5° C. A flow rate of 0.5 ml/min was maintained. Three ml fractions were collected. The optical density of the eluate was read at 278 mμ. Alternatively, the color developed by the Lowry method (97) was read at 750 mμ. Peaks of the three fractions F_1, F_2, and F_3 were collected. F_1 was dialyzed against 0.25 N HCl for 3-5 hours. Then the three fractions were treated with ammonium Reineckate to precipitate the histones. The precipitates were resuspended in minimal amounts of water and dialyzed against 0.25 N HCl to remove the Reineckate. Later, the histones were dialyzed against the desired buffer. The chromatographic fractions F_1, F_2, and F_3 from zinc deficient and control rats were analyzed by disc electrophoresis.

CHAPTER III

RESULTS

A. Evidence of Zinc Deficiency in the Rats Examined

Rats fed the zinc-depleted diet showed reduced growth, inflammed lesions of the skin around the mouth, nose, and the eyes as well as the paws. The hair became coarse, and certain areas especially the shoulders were denuded. In some severely deficient rats weakness was followed by death. The growth curves of deficient and control rats started to deviate during the first week of the experiment, and continued to do so thereafter (Fig. 2). Within twenty to forty days, when the rats were being killed the difference in the mean body weights of the two groups was statistically highly significant ($t = 5.065$ for $n = 18$, $P < 0.01$). Furthermore, Table 1 shows that zinc content of hair in the deficient rats decreased drastically. While, in the control the level of zinc in hair either increased or decreased slightly depending on zinc intake.

B. The Histone Solubility Characteristics, Yields and Purity

The histones were soluble in water, acidic solutions, or buffers at pH less than 7. Upon addition

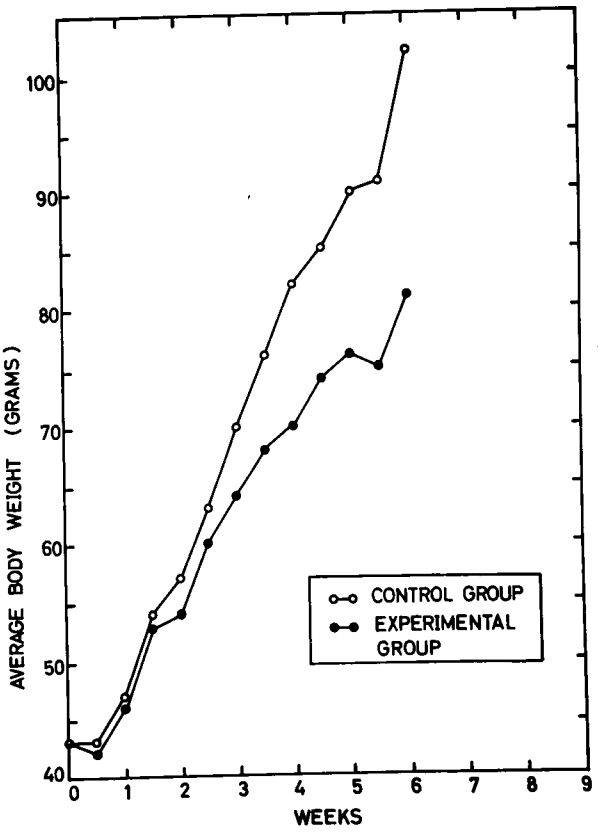

FIGURE 2. A TYPICAL GROWTH CURVE OF ZINC DEFICIENT AND CONTROL RATS (SERIES IX).

Table 1

Hair Content of Zinc* (µg/g) in Zinc Deficient and Control Rats

Series No.	Days**	Control rats		Deficient rats	
		Initial	Final	Initial	Final
9	20	155 ± 4.6	187 ± 23.0	154 ± 14.5	127 ± 24.2
11	20	179 ± 10.5	169 ± 7.6	185 ± 9.5	142 ± 17.8
14	30	162 ± 12.2	156 ± 17.1	139 ± 9.0	96 ± 27.6

*The mean and the standard deviation are reported.

**Days refer to the period between initial and final hair clipping of the rats.

of dilute NH_4OH or raising the pH to eight some of the histones precipitated. The part remaining in solution could be recovered at pH 10.0 by the addition of three volumes of 98 % ethanol or acetone. 20 % trichloroacetic acid (TCA) precipitated the histones completely. Furthermore, the histone preparations dissolved in the Mirsky reagent (1.88 M H_2SO_4 - 0.33 M $HgSO_4$).

The overall yield of histones from wet tissues was 0.05 %. A range in yield of 0.04 - 0.07 % occurred.

Paper electrophoresis of histone preparations at pH 8.6 showed two bands. One, that did not migrate probably because the buffer pH approximated the isoelectric point of the histone fraction, and another that migrated toward the cathode.

6. <u>The Number and Pattern of Histone Bands on Disc Electrophoresis</u>

A maximum of twelve bands could be observed (Fig. 3) in the DFP treated preparations. Six of these bands were prominent. The remaining ~~seven~~ fast mobility bands stained faintly, and compared to the other six, represented a minor proportion of all histones. Four of the six prominent bands accounted for more than 80 % of the total.

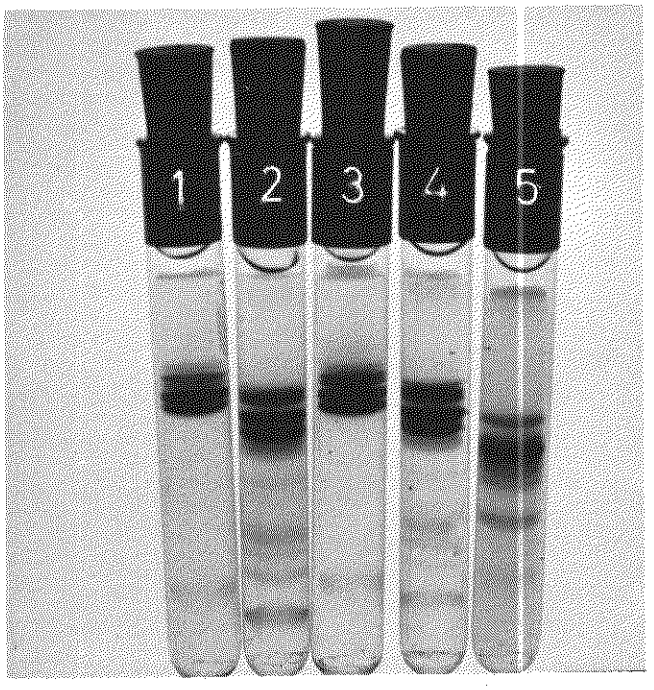

FIGURE 3. DISC ELECTROPHOREGRAM OF HISTONES.

1. EIA treated histones from normal rats.
2. DFP treated histones from normal rats.
3. EIA treated histones from deficient rats.
4. DFP treated histones from deficient rats.
5. Histones from calf thymus.

2 and 4 show twelve bands.

1 and 3 show six bands.

The direction of migration is from the top to the bottom of the tubes.

Occasionally some bands were absent from the histone preparations. However, no extra bands could be detected. In EIA treated preparations the six fast mobility bands were either reduced or absent (Fig. 3). The electrophoretic pattern was constant under the specified conditions, such that relative mobilities of the individual histone bands could be calculated. Bands were numbered starting with the slowest moving band first. The distance travelled by the band number 4 was defined as unity, and the other bands referred to it (Table 2).

D. The Effect of Protease Inhibitors on Histone Preparations

Enzymatic degradation of histones was detectable in pilot experiments for the isolation of nuclei. On disc electrophoresis numerous new bands appeared concomitant with diminution of known major bands. Furthermore, the degraded histones could not be precipitated by 20 % TCA.

EIA and DFP are alkylating agents, hence their use as known enzyme inhibitors. Cathepsins or nuclear proteases are believed to be deactivated by the two inhibitors. In fact, it was not until the introduction of DFP and EIA treatment that successful results were achieved as evidenced by constancy in number and pattern of histone disc electrophoretic bands.

Table 2

Relative Mobility of Histones on Disc Electrophoregrams

Direction of migration →	Histone Band Number											
	1	2	3	4	5	6	7	8	9	10	11	12
Relative mobility	0.75	0.81	0.86	1.00	1.11	1.19	1.39	1.49	1.89	2.03	2.17	2.52
Stain Intensity	**	***	***	***	***	**	*	*	**	**	**	*

The relative mobilities are averaged. Variations of ± 0.05 occurred.

Stain intensity of a band was described as:

* Faintly stained; ** intermediate; *** strongly stained.

However, EIA treated preparations showed the absence of fast mobility bands /7-12/ observed in DFP treated histones (Fig. 3). Moreover, upon fractionation of histones treated with EIA on C.M. Cellulose, and subsequent disc electrophoresis, the F_1 peak showed most of the fast mobility bands (Fig. 4).

The cause of the interesting difference between the effects of the two inhibitors is unknown. Recent reports that thiol groups exist in histones (72) may explain the discrepency partly, since DFP and EIA may be expected to have different affinities to these thiol groups.

E. Comparison of Rat Liver Nuclear, DNP and Calf Thymus Histones

Starch gel electrophoresis of rat liver nuclear and calf thymus histones showed identical behavior. Three zones of the same mobility appeared over the pH range 4.5 - 4.8. However, the two types of histones on disc electrophoresis differed completely in number, mobility and intensity of staining of bands (Fig. 3). Disc electrophoregrams of histones obtained directly from nuclei or indirectly from DNP showed some similarity (Fig. 5).

FIGURE 4. DISC ELECTROPHOREGRAMS OF HISTONE FRACTIONS.

Tubes number 1, 3 and 5 show F_1, F_2 and F_3 histone fractions from control rats respectively.

Tubes number 2, 4 and 6 show F_1, F_2 and F_3 histone fractions from deficient rats respectively.

F_1 shows nine bands.

F_2 and F_3 represent four bands each.

Direction of migration is from the top of the tube to the bottom.

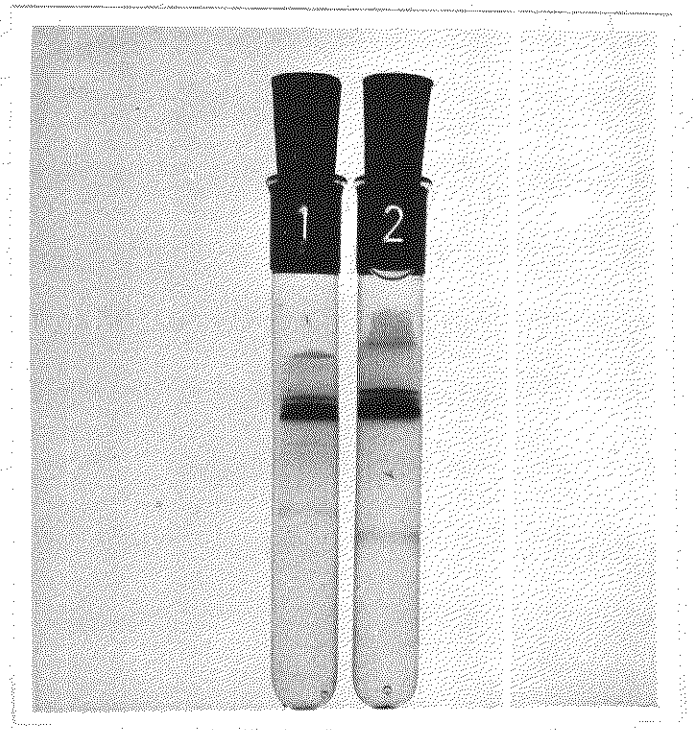

FIGURE 5. DISC ELECTROPHOREGRAM OF NUCLEAR AND DNP HISTONES.

1 represents DNP histone.

2 represents nuclear histone.

The electrophoresis of these samples was carried out at 8 ma/tube for 25 minutes.

Direction of migration is from top of the tube to the bottom.

F. Comparison of Histones from Deficient and Control Rats

Histones from zinc deficient and control rats showed similar disc electrophoretic patterns. Some variation in the thickness of individual bands explained perhaps by differences in protein content, was observed especially in bands number 3 and 5.

The gels were scanned and areas under the absorbance curves of six prominent bands /1-6/ measured. The data in Tables 3 and 4 represent relative concentration of histones in individual bands expressed as percent of total. The statistical analysis of these figures showed that histones treated with DFP behaved unlike EIA treated preparations. In DFP treatment the variation of band number 5 in deficient as compared to control rats was statistically significant. In EIA preparations zinc deficient and control histones did not appreciably vary.

G. Chromatographic Behavior of Histones

Histones from zinc deficient and control rats were compared on C.M. Cellulose columns. To accomplish this, equal amounts of histones were added and eluted under similar conditions. The elution patterns were identical (Figs. 6, 7). In both, some protein was not

Ta

Comparison of Histone Disc Electropho

Series No.	No. of rats	Band N					
		1		2		3	
		D	C	D	C	D	C
IX	4			22	17	16	13
IX	4			17	15	13	10
IX	6	9	6	12	10	18	12
XI	12	8	8	17	10	14	13
Mean ± S.E.		8.5 ±.50	7.0±1.00	17.0±2.06	13.0±1.81	15.3±1.87	12.0±0.
S.E.		1.12		2.77		1.35	
t		0.446		1.455		1.200	
p		p > 0.50		p > 0.10		p. > 0.10	

The figures in the table represent areas under the curve of absorb
total.

The t values test the signifi cance of differences of means between

S.E. is standard error. All calculations are corrected for small

D stands for deficient, C for control.

le 3

etic Bands in DFP Treated Preparations

mber						
	4		5		6	
	D	C	D	C	D	C
	28	31	11	24	23	17
	32	26	22	29	9	15
	25	21	23	37	13	15
	44	46	18	28		
1	32.3±4.18	31.0±5.41	18.5±2.74	29.5±2.83	15.0±4.18	15.7±0.71
	6.84		3.89		4.18	
	0.190		2.736		0.164	
	p > 0.50		0.05 > p > 0.02		p > 0.50	

nce recordings of scanned bands. The areas are expressed as % of

zinc deficient and control histones.

mples.

Comparison of Histone Dis

Series No.	No. of rats	1		2		
		D	C	D	C	D
IX	4	8	10	10	17	12
IX	6	11	6	14	19	13
XI	10			24	28	17
XIV	10	7	5	30	23	19
Mean ± S.E.		8.7±1.23	7.0±1.53	19.5±4.56	21.8±2.45	15.3±1.69
S.E.		1.98		5.22		2.
t		0.857		0.441		
p		p > 0.10		p > 0.50		p >

For explanations refer to Table 3.

[redacted]

Electrophoretic Bands in EIA Preparations

Band Number						
	4		5		6	
C	D	C	D	C	D	C
12	38	32	33	29		
13	35	34	26	21	3	7
20	35	35	24	18		
13	28	26	23	39		
14.5±1.87	34.0±2.12	31.8±2.00	26.5±2.24	26.8±4.69		
1	2.93		5.22			
285	0.752		0.058			
.50	p > 0.10		p > 0.50			

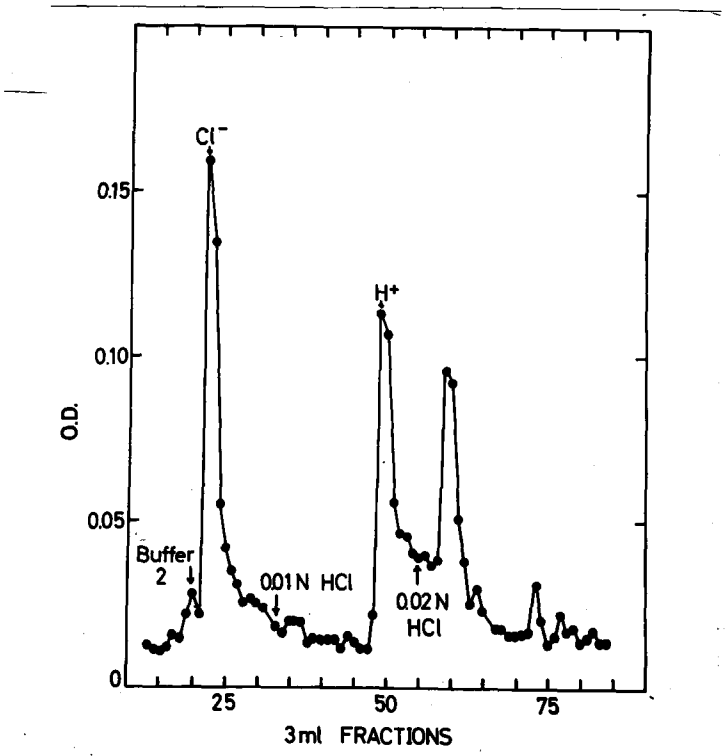

FIGURE 6. C.M. CELLULOSE CHROMATOGRAPHY OF HISTONES FROM ZINC DEFICIENT RATS.

The absorbance at 278 mμ was read.

10 mg of sample was applied to 20 x 1 columns.

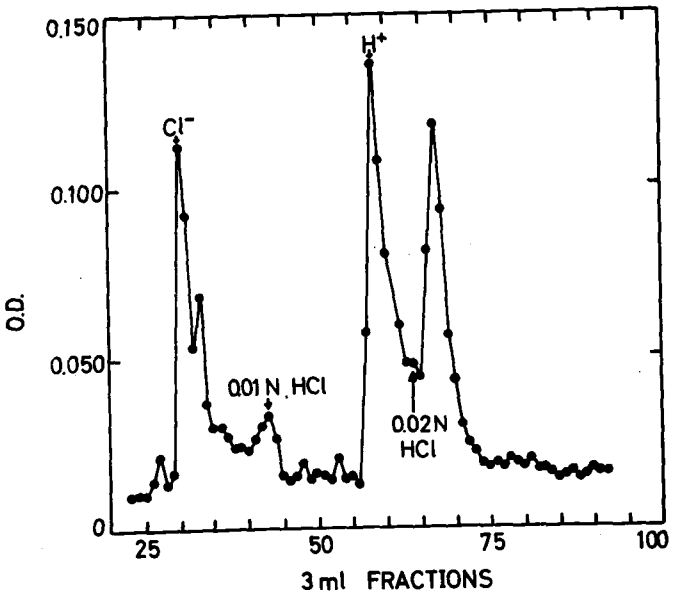

FIGURE 7. C.M. CELLULOSE CHROMATOGRAPHY OF HISTONES FROM CONTROL RATS.

The absorbance at 278 mμ was read.

10 mg of sample was applied to 20 x 1 columns.

retained by the adsorbent column indicating the presence of less basic components. The 278 mμ absorption peaks correlated with those at 750 mμ (Fig. 8), obtained by treatment with the Folin-Ciocalteu reagent (97).

The disc electrophoretic patterns of chromatographically separated fractions F_1, F_2 and F_3 from zinc deficient and control rats were identical (Fig. 4). The F_1 fraction contained 9 bands, F_2 and F_3 showed four bands each. Fast mobility bands appeared only in F_1. Fractions F_2 and F_3 showed the bands number 2 to 5. The F_2 differed from F_3 in the intense staining of zones 4 and 5 (Fig. 4).

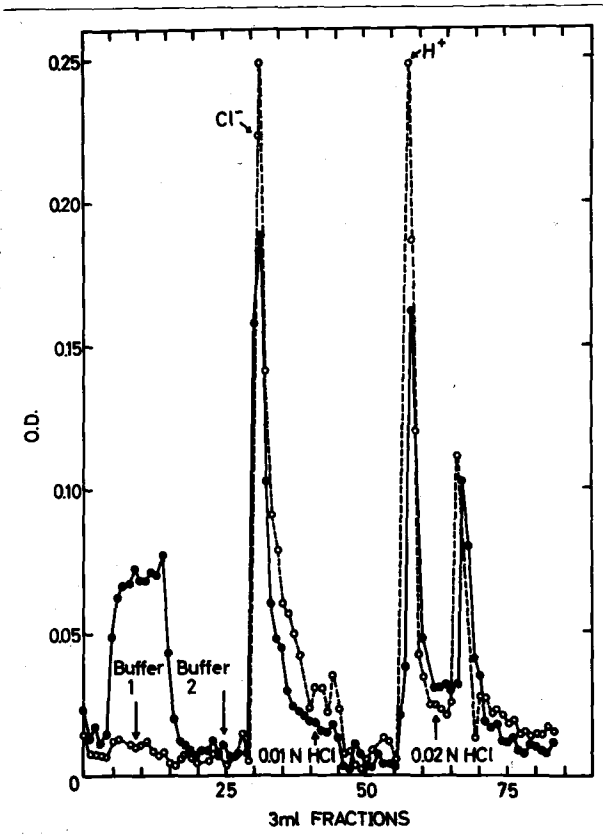

FIGURE 8. C.M. CELLULOSE CHROMATOGRAPHY OF RAT LIVER HISTONES.

●——●——●——● Absorbance at 278 mμ.

o——o——o——o Color developed by Folin-Ciocalteu reagent read at 750 mμ.

The scale on the right is in 0.100 units.

12 mg of histone was applied.

CHAPTER VI

DISCUSSION AND CONCLUSION

Histones are postulated to be genetic repressors. Evidence that histones are capable of inhibiting DNA replication (86) and of m-RNA synthesis (82, 83) supports such a hypothesis. Further support for a repressor function is provided by experiments in which it was shown that acetylation of histones prior to stimulation of m-RNA synthesis in activated lymphocytes eliminated the repressor action (69).

In zinc deficiency protein synthesis and growth are impaired. RNA levels are lowered (28), unlike DNA which appears to be unaffected (100). Inhibition of transcription of genetic information from DNA to RNA and subsequently to protein by a repressor might explain decreased RNA levels and lowered protein synthesis. Hence, the effect of zinc depletion on the behavior of histones is of considerable interest. Detection of changes of type or concentration of nuclear histones might explain in part the molecular basis of the effects of low levels of zinc and strengthen further the histone-repressor hypothesis.

Two fundamental requirements had to be fulfilled in order to obtain a successful treatment of the problem.

Severe zinc deficiency had to be induced in a suitable animal and histones had to be isolated in pure form. The rat was selected because it is susceptible to zinc deficiency. The liver as the major site of protein synthesis would be expected to show metabolic disturbances related to this function. Further, the liver contains substantial amounts of zinc. Therefore, a severe limitation on intake of zinc should result in the appearance of adverse effects in the liver caused by or related to zinc deficiency.

Zinc was almost completely removed from the food and precautions were taken so that no zinc other than provided in the diet was available to the rats. The impaired growth and various physical signs of deficiency gave evidence that severe zinc deficiency existed.

The preparation of histones from the nuclei was preferred because basic proteins are known to occur in the microsomes (101) and perhaps elsewhere. Proteolytic degradation was kept minimal by rapid isolation and purification methods carried out at low temperatures and by the use of protease inhibitors. The DNP histones used in earlier experiments were subject to contamination in addition to enzymatic hydrolysis during the longer period of isolation. Hence, DNP preparations were abandoned and nuclear histones used throughout the experiments.

The mode of preparation, solubility, electrophoretic behavior, and adsorption to and elution from C.M. Cellulose cation exchange columns shows that the isolated products were nuclear basic proteins. The differences in number, mobility and pattern of disc electrophoregrams of calf thymus and rat liver histones demonstrate that histones are a complex of closely related proteins. Although up to 12 bands were detected in the acrylamide gels, the number of individual histones may be smaller, some of the minor bands may represent decomposition products. Treatment of histones with urea or electrophoresis at lower pH produces more bands. A limited number of histones restricts repressor action to changes in histone type or amount. Hence levels of histones in the nucleus may be significant (33, 85).

Histones from zinc deficient and control rats compared electrophoretically showed a significant changes in DFP treated preparations. However, EIA preparations failed to show such a difference. The demonstration of statistically significant differences despite the small number of assays indicates that histones undergo changes during zinc deficiency. The finding is compatible with a histone-repressor hypothesis. However, the possibility that disturbance of histone synthesis like that of other proteins occurs cannot be excluded.

The chromatographic behavior on C.M. Cellulose of histones from normal and deficient rats was identical. Resolution by means of other chromatographic media may be more successful and should be attempted. Immunoelectrophoretic studies seem worth doing.

In conclusion, histones from liver in zinc deficient rats undergoes a significant change detectable by disc electrophoresis. The cause for the discrepency between the DFP and EIA treated histone preparations remains to be established. It is possible that apart from a protease inhibiting effect these alkylating agents combine with histones chemically.

SUMMARY

1. Liver nuclear histones from zinc deficient and control rats were isolated and compared by electrophoresis on acrylamide gels and by chromatography on carboxymethyl cellulose.

2. Histone preparations in which diisopropylfluorophosphate (DFP) was used to inhibit proteolytic degradation differed from those in which ethyliodoacetate was used.

3. In the presence of DFP, histones isolated from liver nuclei of zinc deficient rats showed statistically a significant change in relative concentration of major histone fractions as compared with similar preparations from liver nuclei of control rats, when subjected to electrophoresis on acrylamide gels at pH 4.3.

4. Carboxymethyl cellulose chromatography failed to demonstrate differences between the deficient and control groups.

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LIVER NUCLEAR HISTONES OF ZINC-DEFICIENT RATS

ARSLANIAN

LIVER NUCLEAR HISTONES OF ZINC-DEFICIENT RATS

By

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LIST OF ABBREVIATIONS

C.M. Cellulose	- Carboxymethyl cellulose
DFP	- Diisopropylfluorophosphate
DNP	- deoxyribonucleoprotein
DNA	- deoxyribonucleic acid
EDTA	- ethylenediaminetetraacetic acid
EIA	- ethyl iodoacetate
RNA	- ribonucleic acid
m-RNA	- messenger ribonucleic acid
ma	- milli amperes
p.p.a.	- parts per million
r.p.m.	- revolutions per minute
x g	- times gravity

CHAPTER I

INTRODUCTION

A. Zinc Deficiency

In 1869 Raulin (1) recognized zinc as an essential element. Since then, a great deal of evidence substantiating the universal importance of this micronutrient has accumulated. States of zinc deficiency are described in *Aspergillus niger* (1), *Neurospora crassa* (2-4), corn (5), tomato (6), and other plants. In the animal kingdom, indisputable evidence for the specific effects of zinc deprivation in the rat was presented by Todd *et al.* (7), and the Wisconsin group (8-10). Subsequently, zinc deficiency syndromes were documented for swine (11), chicken (12, turkey (13), calves (14), lambs (15), and the Japanese quail (16).

Despite the overwhelming evidence for the existence of zinc deficiency states, the nature of metabolic disturbances it causes remains unknown. The subcellular distribution of zinc in the rat liver follows closely the intracellular localization of known zinc metalloenzymes (17). These have been shown to lose activity when their zinc is bound with complexing agents, and to regain activity after replacement of the zinc (18, 19).

Hence, it may be expected that zinc deprivation should result in loss or reduction of activity of most zinc metalloenzymes. In fact, alcohol dehydrogenase was absent in zinc-deficient mycelia of Neurospora crassa (3-4). Subnormal levels of intestinal and kidney alkaline phosphatase activities in the rat were reported (10, 20-21). Also, bone alkaline phosphatase activity was lowered in zinc deficient turkeys (22). However, carbonic anhydrase, and lactic dehydrogenase, both zinc dependent enzymes, were found to be unaffected by zinc deficiency in rats (23), and chicken (12). Furthermore, in the male genital tract which accumulates high concentrations of zinc, the amounts of the metal do not reflect the activities of known zinc metalloenzymes (24).

Impaired growth in plants and animals during zinc deficiency has indicated that protein synthesis is one of the biochemical loci affected. Changes have been found in the levels of plasma proteins of zinc-deficient rats (9), chicks (25), and the Japanese quail (26, 27). Lower protein content in zinc-deficient mycelia of Neurospora crassa (3, 4), and tomato (6) has also been reported.

Zinc has been suggested to have a role in protein, m-RNA and DNA syntheses. Schneider and Price (28) have hypothesized that decreased levels of RNA may be the cause of growth inhibition during zinc deprivation. In fact,

investigations of the effect of zinc deficiency upon RNA, and protein synthesis, in Euglena gracillis (29), Rhizopus nigricans (30), and Mycobacterium smegmatis (31, 32) have described a general pattern. The metabolic disturbances associated with zinc deficiency, they believe, starts with the arrest of RNA formation; soon protein synthesis stops. Later, the rising total nitrogen and DNA levels associated with growth come to a standstill. Addition of zinc to the culture media stimulates RNA and protein synthesis (30). Further, it is of interest that in experiments performed by Fox and Harrison (27), Actinomycin D, the inhibitor of DNA primed m-RNA formation, influenced zinc-deficient birds only, during short periods of fasting. This implies disturbed m-RNA formation during zinc deficiency. However, injection of growth hormone, which stimulates m-RNA formation, caused retention of nitrogen but no weight gain in deficient rats, a finding that does not support direct m-RNA involvement.

Nuclear basic proteins, the histones are known to be involved in DNA dependent RNA synthesis. As an example, histones in 100 µg/ml concentration activated de novo synthesis of lactic dehydrogenase, a zinc metalloenzyme from embryonic chick brain in tissue culture. While, at 400 µg/ml complete inhibition of the enzyme synthesis occurred (33). Since histones could inhibit the formation

of a zinc-dependent enzyme *in vitro*, and probably other proteins *in vivo*, the possibility that during zinc deficiency synthesis of some key metalloenzymes were blocked by histones needs to be considered. If histones behaved as repressors, definite changes in histone concentration or type might be expected during zinc deprivation. For these reasons a study of histone behavior was made in zinc-deficient rats and compared with that of controls receiving appropriate quantities of zinc.

B. Histones

The histones, basic proteins of molecular weight 8000-130000, are found in close association with the DNA of the nucleus of somatic cells (34). Their metabolic role is not known. However, keen interest was aroused in them by Stedman *et al* (35) who suggested a genetic regulatory role for them. Significant findings concerning heterogeneity, species and organ specificity and nature of interaction of histones with the DNA have been described (36).

The heterogeneity of histones is well established. Differential extraction and precipitation, ion exchange chromatography, gel filtration, electrophoresis (37,38), and countercurrent distribution (39) techniques have been used to separate histones into three main fractions;

arginine-rich (F_1), moderately lysine-rich (F_2), and lysine-rich (F_3). Each has been further subfractionated. Starch gel electrophoresis (40, 41) of whole, as well as chromatographically separated fractions (42, 43) of Histones yields multiple bands. Extensive heterogeneity was revealed by the use of polyacrylamide gels (40, 44). Moreover, Murray (45) demonstrated that histones prepared by these widely different methods exhibited heterogeneity. The present belief is that there are a limited number of histones which have remarkable similarities in electrophoretic patterns (40, 46), amino acid compositions (47, 48) and NH_2-terminal amino acids (49, 50). Comparisons of these characteristics were made with calf thymus histones which have been most completely studied.

Species and organ specificity of histones have been investigated. Neelin (40, 51) and Hnilica (42) isolated a unique histone from chicken erythrocytes. Differences from various organs of chicken (40) and rats (42) have also been described, as have slight differences between histones of the same organs of the rat, guinea pig, and the rabbit (52). However, histones from mouse brain, and liver (53), calf thymus, liver and kidney (50), and rat brain, liver and kidney (42) showed no detectable differences.

An interesting succession of histone types during spermatogenesis in the squid Loligo (54) and fertilization in the snail Helix aspera (55) were reported. Concomitantly, newborn and adult tissues from chicken showed minor differences (46). Also, slight but consistent differences in the histones of young rat liver, and brain were detected (52). Microorganisms grown under different conditions were investigated and significant differences in the histones were observed (56). Small differences between normal and neoplastic tissues have been also described (34).

C. Histone Metabolism

The site of biosynthesis of histones is unknown. The nucleus has been suggested (54, 57) although during spermatogenesis in the grasshopper, protein-bound labeled arginine appeared first in the cytoplasm of the sperm nucleus. Originally it was believed that histone and DNA syntheses occurred simultaneously (58, 59). Recent evidence indicates that histone synthesis is an independent process, and may take place before DNA synthesis (60, 61). Actinomycin D, the known m-RNA inhibitor, causes a decrease in the incorporation of labeled amino acids into histones (62). Moreover, inhibition of histone synthesis by puromycin indicates that histone biosynthesis is like that of other proteins (63, 64).

Histones undergo a slow but definite turnover (65, 53), although in avian erythrocytes and reticulocytes no turnover could be detected (66). C^{14} lysine is incorporated into the peptide structure of lysine-rich histone fractions (67). Furthermore, labeled amino acids are incorporated at different rates into histone fractions isolated from calf thymus nuclei (62, 64).

Methylation and acetylation of histones, which modify histone-DNA interaction, can occur both *in vivo* and *in vitro* (68, 62). These processes are not inhibited by puromycin (63). Recent evidence indicates that during gene activation in human lymphocytes, acetylation of histones (69) takes place just before the rise in RNA levels. Phosphorylation of histones also occurs (70). Serine phosphate has been identified as the site (71). Thiol groups have also been reported in histones (72).

The concentration of histones in the nucleus was assumed to be equivalent to that of DNA (73). However, Umana *et al* have shown that in nondividing stable cells, histone concentration is twice that of DNA. Starvation lowers the histone:DNA ratio to 1.65. In tumors the ratio is about one (74). The amount of histones to DNA in adult tissues varies (75).

Basic groups of histones interact with phosphate groups of DNA primarily through nonspecific ionic linkages

(76, 77). The ease of dissociation of nucleohistone complexes by acidic or high ionic strength solutions has been presented as evidence (77). The results of analyses of tryptic finger prints indicate that basic amino acids in histones are not found in a regular order as are the phosphate groups of DNA (45, 78). Furthermore, the interaction of histones with DNA seems to be size and structure-dependent (79). Nucleohistone association and dissociation studies (80, 81) have shown that a lysine-rich histone fraction specifically associates with a DNA moiety rich in guanine and cytosine.

D. Histones in the Biochemistry of the Nucleus

Histones were shown to be involved in several reactions taking place in the nucleus. Among these are: synthesis of DNA-dependent RNA, of DNA and of nuclear ATP.

In isolated thymus nuclei, addition of histones inhibits DNA-dependent RNA synthesis (82, 83). While, enzymatic removal of histones with trypsin or acetylation, enhances it. Pea embryo native chromatin or reconstituted nucleoprotein (histone:DNA, 2:1) is inactive in supporting DNA-dependent RNA synthesis (73, 84). Selective removal of histones from reconstituted nucleoprotein stimulates the incorporation of labeled RNA precursors.

The degree of inhibition caused by different histone fractions is unknown (73, 82, 84). Furthermore, the concentration of histones in relation to DNA may be significant (33, 85).

The interaction of histones with DNA (86) and nuclear ATP (87) syntheses are less well understood. However, histones inhibit both of the above mentioned reactions. Hence, it is expected that most phosphorylating and energy requiring processes in the nucleus may be adversely affected.

CHAPTER II

MATERIALS AND METHODS

A. Induction of Zinc Deficiency in Rats

Weanling rats, all male, of a local Sprague-Dawley strain were sorted at random into experimental and control groups. The rats were housed in stainless steel cages, and were pair-fed the appropriate diets after a twenty-four hours fast. Deionized water was provided *ad libitum*. The diet was based on that of Forbes and Yohé (88) with the following modifications: the protein was changed to 80 % casein (EDTA-purified as described in (89)) and 20 % gelatin. Cellulose and chlortetracycline were omitted. Instead of glucose, sucrose and dextrin served as carbohydrate sources. 1500 I.U. Vitamin D, and 12000 I.U. Vitamin A, and 1.0 g Tocopherol were added per 4.5 kg batch. The $CaHPO_4$ used in the salt mixture was prepared from Na_2HPO_4 and $CaCl_2$ by isoelectric precipitation to decrease zinc contamination found in commercially available $CaHPO_4$.

The zinc content of the food was measured with the aid of dithizone (90) or atomic absorption spectrophotometry (91), after digestion with sulfuric, nitric and perchloric acids (89). The zinc-depleted diet contained

less than two p.p.m. zinc. The controls received the same diet except that $ZnCO_3$ was added to raise the zinc level to 20 p.p.m.

The zinc content of hair was measured as a supplementary method for assessing zinc deficiency (89). Hair was clipped from the abdomen, washed, digested and analyzed by atomic absorption spectrophotometry (89).

The rats were weighed twice each week. As soon as severe zinc deficiency symptoms developed the experiment was terminated. This occurred twenty to forty days after starting diets.

B. Preparation of Rat Liver Nuclei

Rats fasted for twenty-four hours were anesthetized with ether, the abdominal and thoracic cavities were opened. Blood was withdrawn with a syringe from the left ventricle. A catheter was introduced into the vena cava and the liver was perfused *in situ* with ice-cold 0.14 M NaCl to remove blood. Perfusion was continued until the liver became gray. All subsequent operations were carried out at 5° C or below.

The liver was rinsed with saline, blotted with filter paper, and one gram portions were minced with scissors and homogenized in twenty volumes of 0.25 M sucrose, 3 mM $CaCl_2$. Either 1 mM diisopropyl fluorophosphate

(DFP) or 0.1 mM ethyl iodoacetate (EIA) were added just before homogenization. DFP, and EIA were used to inhibit enzymatic hydrolysis of histones known to occur in the nucleus upon homogenization of the rat liver. A close fitting teflon pestle with serrated edge, and a Potter Elvehjem glass homogenizer were used. After numerous trials 6-8 strokes, 40 seconds and 3500 r.p.m. for the rotor were found to yield maximal number of intact nuclei. The homogenate was passed through four layers of cheese cloth, and centrifuged for ten minutes at 1000 x g. The crude nuclear sediment was resuspended in the homogenizing solution (1 g/10 ml) and was centrifuged as above. The pellet obtained was suspended as described by Chauveau (92) in 2.1 M sucrose using a loosely fitting teflon pestle with the glass homogenizer. The nuclear suspension corresponded to 5 g of the original tissue per 100 ml of 2.1 M sucrose solution. Heads 21 or 30 of the model L Spinco preparative ultracentrifuge were used for 75 minutes at 50000 x g calculated for the bottom of the centrifuge tube. Samples of the nuclei prepared were stained by hematoxylin hydrochloride and examined with the light microscope. The nuclear preparations contained little detectable contamination (whole cell or debris). Yields were low.

C. Preparation of Histones by Isoelectric Precipitation

Initially a procedure based upon isoelectric precipitation of histones was tested. Two histone fractions from the rat liver were obtained by a modification of Daly and Mirsky method (93). The nuclei were extracted with 0.25 N HCl and the clear supernatant solution obtained after centrifugation was titrated to pH 8 with 1 N NaOH. Turbidity, indicating aggregation, appeared first at pH 6 and at about pH 8 a cloudy, white precipitate formed. No further precipitation was observed upon raising the pH. After the first histone fraction was collected, the pH of the supernatant solution was raised to 10.0 and three volumes of 98 % ethanol were added. A white precipitate, the second histone fraction, was formed. The isoelectric precipitation procedure was abandoned because both histone fractions obtained were contaminated by other proteins having isoelectric points in the same pH range. Also, alkaline treatment of histones caused denaturation, hence loss of solubility.

D. Preparation of Deoxyribonucleoprotein (DNP)

A second procedure applied to isolation of histones depended upon separation of deoxyribonucleoprotein. All steps described previously in the high speed differential centrifugation of nuclei were followed. The nuclear

pellet was extracted with 2 M NaCl (50) for 5 hours. The supernatant solution was decanted and centrifuged 2 hours at 2000 x g. The new supernatant solution containing the DNP was diluted to a concentration of 0.15 M NaCl. The DNP precipitate was collected by centrifugation.

E. Purification of Histones

The clean nuclei or DNP were extracted with 0.25 N HCl for two to three hours. The extract was centrifuged and the clear supernatant solution containing the histones was purified by the method of Lindh and Brantmark (38).

The acid extract was treated with a saturated solution of freshly prepared ammonium Reineckate until complete precipitation occurred. The histone Reineckate was washed with an appropriate volume of 0.05 M Tris HCl pH 8.4 buffer. An equal volume of saturated Reineckate was added, and the precipitate was collected by centrifugation. The supernatant discarded contained proteins with isoelectric points below pH 7, together with low molecular weight basic contaminants. This step was repeated twice. The precipitate was treated with acetone HCl (98 parts acetone : 2 parts concentrated HCl) which had been cooled below -5° C, and centrifuged at -20°C. The precipitate containing the histones was extracted with β-alanine-acetate buffer at pH 4.5, or acetate buffer at pH 4.2.

An insoluble greenish precipitate remained. The flowsheet summarizes the different steps (Fig. 1).

F. Disc Electrophoresis of Histones in Acrylamide Gels

Disc electrophoresis was performed according to Reisfeld (94) in 7.0 % acrylamide gels. A combination of large and small pore gels at pH 6.7 and pH 4.3 respectively was used to produce the molecular sieving and electrophoretic effects required to resolve mixtures of closely related proteins (95, 96). Unless otherwise specified, a 6 ma constant current was applied for 65 minutes per tube. 7.5 cm long and 0.5 cm internal diameter glass running tubes were used. The gel stacked at pH 5.0 and ran at pH 4.3. The buffer used for electrophoresis was 0.35 M β-alanine-acetate pH 4.5. Under these conditions histones showed excellent resolution.

A volume of solution containing fifty microgram histone samples was applied. Larger protein samples are known to cause aggregation with loss of resolution; furthermore, scanning of thick bands is inaccurate. Protein concentration was measured by the method of Lowry et al (97). A preparation of calf thymus histone* was used for the protein standards.

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*HLY 61 A Worthington Biochemicals.

FLOWSHEET FOR THE PREPARATION OF HISTONES

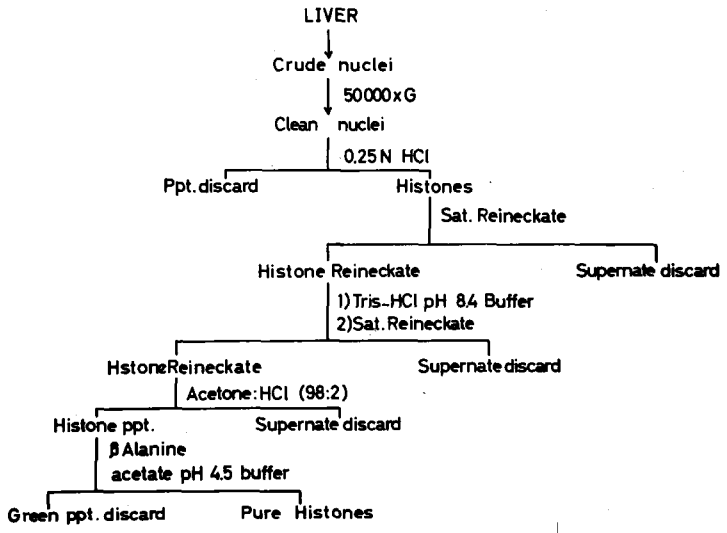

FIGURE I. FLOWSHEET FOR RAT LIVER NUCLEAR PREPARATIONS.

The histone sample was mixed with an equal volume of gel solution composed of 1 part B : 2 parts D : and 1 part E, where B, D and E are (94):

B. 48 ml 1 N KOH, 2.87 ml glacial acetic acid, 0.46 ml of N,N,N',N'-tetramethylethylenediamine in 100 ml water solution.

D. 10 g acrylamide, 2.5 g N,N'-methylenebis-acrylamide in 100 ml water solution.

E. 4.0 mg riboflavin in 100 ml water solution.

Addition of four parts water was omitted from the gel solution because the samples were dilute. Histones applied were either in water or in β-alanine-acetate buffer pH 4.5 or in acetate buffer pH 4.3. After the run, the gels were removed from the tubes with the help of a needle, and a syringe filled with 7 % acetic acid. While a gentle stream of the solution was released, the needle was inserted between the gel and the tube wall, and a complete turn around the gel made.

The gels were immersed in 0.5 % Amido Schwarz 10 B, in 7 % acetic acid, for a minimum of two hours. They were destained either electrophoretically in 7 % acetic acid with currents less than 8 ma per tube, or by diffusion in the same solvent. The latter was preferred because currents as low as 4 ma/tube removed fainter bands. Also development of artifacts is reported (98).

The destained gels were scanned with Aminco Fluoromicrophotometer equipped with a scanner running at a speed of one centimeter/minute. The photometer was corrected to a Moseley model 680 recorder, and the gel patterns were traced. A wratten ND filter 1-10 % and a green filter 58 were used to decrease the intensity of light, and make the use of slit adjustment possible. An unstained section of the gel was selected to adjust the zero absorbance above the baseline. Several recordings were made at different slit widths. The tracing showing the best resolution of bands with the least noise was selected for analysis. Some variation in the optical properties of the gel which could not be avoided gave rise to minor differences in the optical density for the clear areas. Hence, the region of the more prominent bands (4 major, 2 minor) was analyzed only. The curves corresponding to the absorbance of the protein bands on the gel were assumed to have Gaussian symmetry. When two curves overlapped because two close bands could not be completely resolved by the scanner, the missing arms of the two curves were drawn symmetrically with the other. The area falling under the curves was measured with a planimeter. The average of five such measurements was recorded. Each peak was expressed as % of total area. Since the quantity of protein applied to the gel columns

was the same, the several preparations examined could be compared directly by areas under the curves.

G. Starch Gel Electrophoresis

Starch gel electrophoresis in acetate buffers of differing ionic strength and pH was also tested. The best results were obtained with acetate buffer pH 4.8, 0.02 ionic strength. Because at least 500 µg samples were required and resolution as compared to disc electrophoresis was poor, it was discontinued.

H. Paper Electrophoresis

The presence of nonbasic protein contaminants in substantial amounts was excluded by electrophoresis carried out on paper in veronal buffer pH 8.6, ionic strength 0.075 and a current of 2.5 ma/cell for 16 hours. The paper strips were stained with 1 % Amido Schwarz in 7 % acetic acid, and destained in the same solvent.

I. Fractionation of Histones by Carboxymethyl Cellulose

Rat liver histones were fractionated on C.M. Cellulose* columns according to the method of Johnes et al (99). The protein was added to the column in 0.1 M

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*C.M. Cellulose Sigma Chemical Co. medium mesh 0.60 mEq/g.

acetate pH 4.2 buffer. Three fractions were eluted by the use of 0.2 M acetate - 0.42 M NaCl buffer pH 4.2, 0.01 N HCl, 0.02 N HCl respectively. 20 x 1 cm columns were used. The C.M. Cellulose was previously washed with 4.2 pH acetate buffer to remove any ultraviolet absorbing moieties. In order to compare histones from zinc deficient and control rats, identical amounts of samples were added and eluted. Fractionation was carried out initially at room temperature. Later, experiments were performed at 5° C. A flow rate of 0.5 ml/min was maintained. Three ml fractions were collected. The optical density of the eluate was read at 278 mμ. Alternatively, the color developed by the Lowry method (97) was read at 750 mμ. Peaks of the three fractions F_1, F_2, and F_3 were collected. F_1 was dialyzed against 0.25 N HCl for 3-5 hours. Then the three fractions were treated with ammonium Reineckate to precipitate the histones. The precipitates were resuspended in minimal amounts of water and dialyzed against 0.25 N HCl to remove the Reineckate. Later, the histones were dialyzed against the desired buffer. The chromatographic fractions F_1, F_2, and F_3 from zinc deficient and control rats were analyzed by disc electrophoresis.

CHAPTER III

RESULTS

A. Evidence of Zinc Deficiency in the Rats Examined

Rats fed the zinc-depleted diet showed reduced growth, inflammed lesions of the skin around the mouth, nose, and the eyes as well as the paws. The hair became coarse, and certain areas especially the shoulders were denuded. In some severely deficient rats weakness was followed by death. The growth curves of deficient and control rats started to deviate during the first week of the experiment, and continued to do so thereafter (Fig. 2). Within twenty to forty days, when the rats were being killed the difference in the mean body weights of the two groups was statistically highly significant ($t = 5.065$ for $n = 18$, $P < 0.01$). Furthermore, Table 1 shows that zinc content of hair in the deficient rats decreased drastically. While, in the control the level of zinc in hair either increased or decreased slightly depending on zinc intake.

B. The Histone Solubility Characteristics, Yields and Purity

The histones were soluble in water, acidic solutions, or buffers at pH less than 7. Upon addition

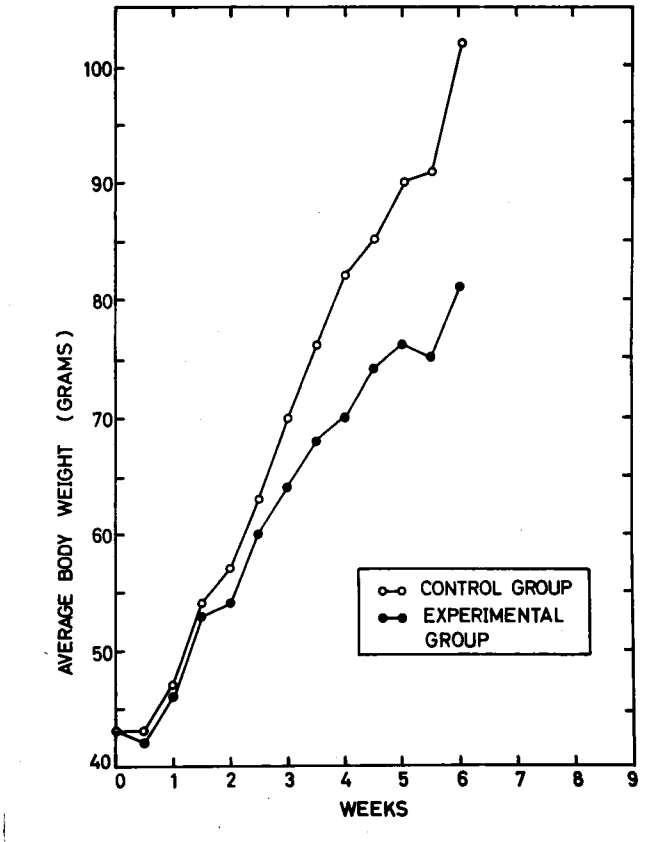

FIGURE 2. A TYPICAL GROWTH CURVE OF ZINC DEFICIENT AND CONTROL RATS (SERIES IX).

Table 1

Hair Content of Zinc* (μg/g) in Zinc Deficient and Control Rats

Series No.	Days**	Control rats		Deficient rats	
		Initial	Final	Initial	Final
9	20	155 ± 4.6	187 ± 23.0	154 ± 14.5	127 ± 24.2
11	20	179 ± 10.5	169 ± 7.6	185 ± 9.5	142 ± 17.9
14	30	162 ± 12.2	156 ± 17.1	139 ± 9.0	96 ± 27.6

*The mean and the standard deviation are reported.

**Days refer to the period between initial and final hair clipping of the rats.

of dilute NH_4OH or raising the pH to eight some of the histones precipitated. The part remaining in solution could be recovered at pH 10.0 by the addition of three volumes of 98 % ethanol or acetone. 20 % trichloroacetic acid (TCA) precipitated the histones completely. Furthermore, the histone preparations dissolved in the Mirsky reagent (1.88 M H_2SO_4 - 0.33 M $HgSO_4$).

The overall yield of histones from wet tissues was 0.05 %. A range in yield of 0.04 - 0.07 % occurred.

Paper electrophoresis of histone preparations at pH 8.6 showed two bands. One, that did not migrate probably because the buffer pH approximated the isoelectric point of the histone fraction, and another that migrated toward the cathode.

C. <u>The Number and Pattern of Histone Bands on Disc Electrophoresis</u>

A maximum of twelve bands could be observed (Fig. 3) in the DFP treated preparations. Six of these bands were prominent. The remaining seven fast mobility bands stained faintly, and compared to the other six, represented a minor proportion of all histones. Four of the six prominent bands accounted for more than 80 % of the total.

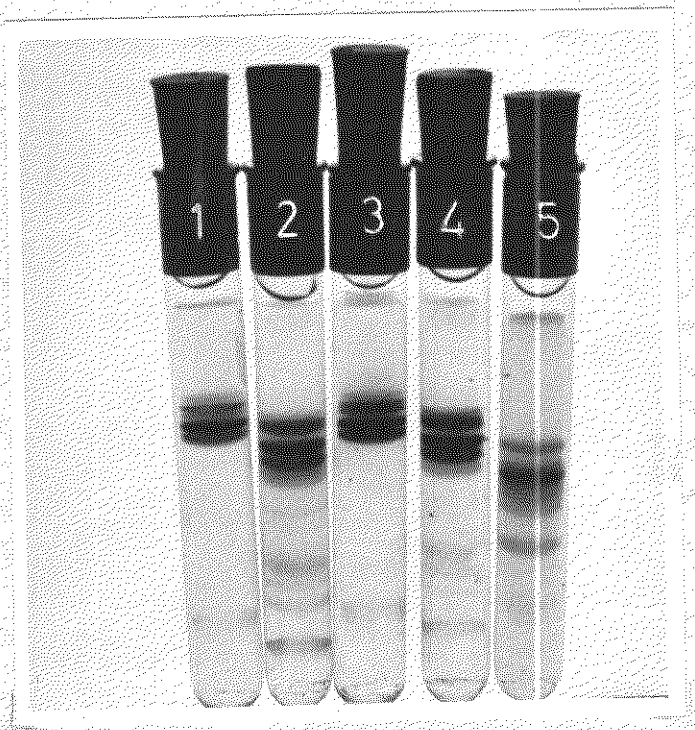

FIGURE 3. DISC ELECTROPHOREGRAM OF HISTONES.

1. EIA treated histones from normal rats.
2. DFP treated histones from normal rats.
3. EIA treated histones from deficient rats.
4. DFP treated histones from deficient rats.
5. Histones from calf thymus.

2 and 4 show twelve bands.

1 and 3 show six bands.

The direction of migration is from the top to the bottom of the tubes.

Occasionally some bands were absent from the histone preparations. However, no extra bands could be detected. In EIA treated preparations the six fast mobility bands were either reduced or absent (Fig. 3). The electrophoretic pattern was constant under the specified conditions, such that relative mobilities of the individual histone bands could be calculated. Bands were numbered starting with the slowest moving band first. The distance travelled by the band number 4 was defined as unity, and the other bands referred to it (Table 2).

D. The Effect of Protease Inhibitors on Histone Preparations

Enzymatic degradation of histones was detectable in pilot experiments for the isolation of nuclei. On disc electrophoresis numerous new bands appeared concomitant with diminution of known major bands. Furthermore, the degraded histones could not be precipitated by 20 % TCA.

EIA and DFP are alkylating agents, hence their use as known enzyme inhibitors. Cathepsins or nuclear proteases are believed to be deactivated by the two inhibitors. In fact, it was not until the introduction of DFP and EIA treatment that successful results were achieved as evidenced by constancy in number and pattern of histone disc electrophoretic bands.

Table 2

Relative Mobility of Histones on Disc Electrophoregrams

Direction of migration →	Histone Band Number											
	1	2	3	4	5	6	7	8	9	10	11	12
Relative mobility	0.78	0.81	0.86	1.00	1.11	1.19	1.39	1.49	1.89	2.03	2.17	2.52
Stain Intensity	**	***	***	***	***	**	*	*	**	**	**	*

The relative mobilities are averaged. Variations of ± 0.05 occurred.

Stain intensity of a band was described as:

* Faintly stained; ** intermediate; *** strongly stained.

However, EIA treated preparations showed the absence of fast mobility bands /7-12/ observed in DFP treated histones (Fig. 3). Moreover, upon fractionation of histones treated with EIA on C.M. Cellulose, and subsequent disc electrophoresis, the F_1 peak showed most of the fast mobility bands (Fig. 4).

The cause of the interesting difference between the effects of the two inhibitors is unknown. Recent reports that thiol groups exist in histones (72) may explain the discrepency partly, since DFP and EIA may be expected to have different affinities to these thiol groups.

E. Comparison of Rat Liver Nuclear, DNP and Calf Thymus Histones

Starch gel electrophoresis of rat liver nuclear and calf thymus histones showed identical behavior. Three zones of the same mobility appeared over the pH range 4.5 - 4.8. However, the two types of histones on disc electrophoresis differed completely in number, mobility and intensity of staining of bands (Fig. 3). Disc electrophoregrams of histones obtained directly from nuclei or indirectly from DNP showed some similarity (Fig. 5).

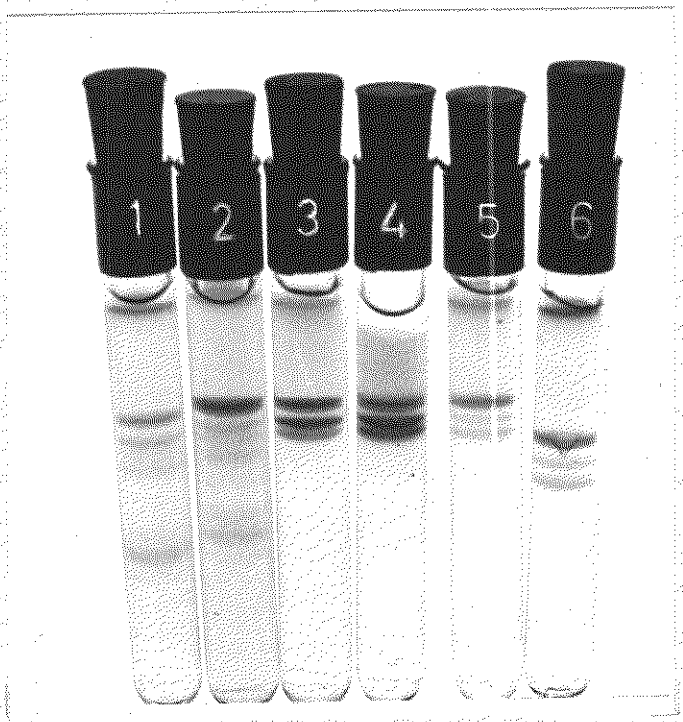

FIGURE 4. DISC ELECTROPHOREGRAMS OF HISTONE FRACTIONS.

Tubes number 1, 3 and 5 show F_1, F_2 and F_3 histone fractions from control rats respectively.

Tubes number 2, 4 and 6 show F_1, F_2 and F_3 histone fractions from deficient rats respectively.

F_1 shows nine bands.

F_2 and F_3 represent four bands each.

Direction of migration is from the top of the tube to the bottom.

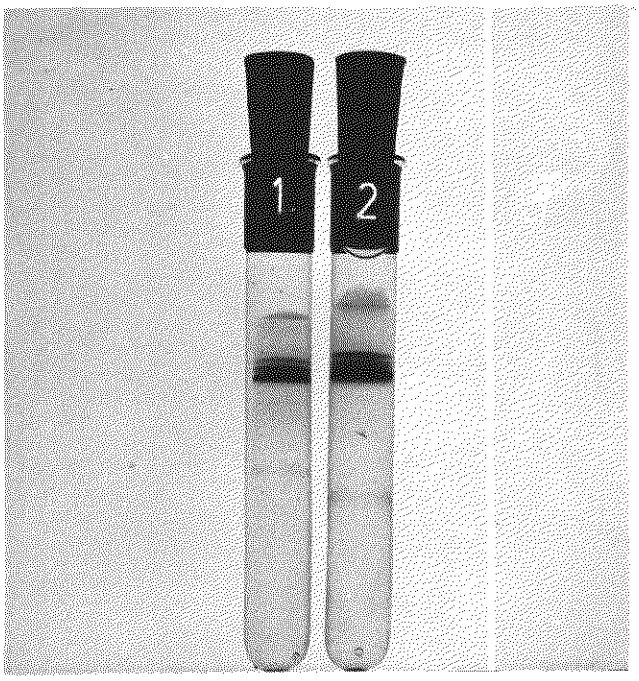

FIGURE 5. DISC ELECTROPHORESIS OF NUCLEAR AND DNP HISTONES.

1 represents DNP histone.

2 represents nuclear histone.

The electrophoresis of these samples was carried out at 8 ma/tube for 25 minutes.

Direction of migration is from top of the tube to the bottom.

F. Comparison of Histones from Deficient and Control Rats

Histones from zinc deficient and control rats showed similar disc electrophoretic patterns. Some variation in the thickness of individual bands explained perhaps by differences in protein content, was observed especially in bands number 3 and 5.

The gels were scanned and areas under the absorbance curves of six prominent bands /1-6/ measured. The data in Tables 3 and 4 represent relative concentration of histones in individual bands expressed as percent of total. The statistical analysis of these figures showed that histones treated with DFP behaved unlike EIA treated preparations. In DFP treatment the variation of band number 5 in deficient as compared to control rats was statistically significant. In EIA preparations zinc deficient and control histones did not appreciably vary.

G. Chromatographic Behavior of Histones

Histones from zinc deficient and control rats were compared on C.M. Cellulose columns. To accomplish this, equal amounts of histones were added and eluted under similar conditions. The elution patterns were identical (Figs. 6, 7). In both, some protein was not

Tabl

Comparison of Histone Disc Electrophore

Series No.	No. of rats	Band Num					
		1		2		3	
		D	C	D	C	D	C
IX	4			22	17	16	13
IX	4			17	15	13	10
IX	6	9	6	12	10	18	12
XI	12	8	8	17	10	14	13
Mean ± S.E.		8.5 ±.50	7.0±1.00	17.0±2.06	13.0±1.81	15.3±1.87	12.0±0.71
S.E.		1.12		2.77		1.35	
t		0.446		1.455		1.200	
p		p >0.50		p >0.10		p. >0.10	

The figures in the table represent areas under the curve of absorbanc
total.

The t values test the signifi eance of differences of means between zi
S.E. is standard error. All calculations are corrected for small sam
D stands for deficient, C for control.

) 3

tic Bands in DFP Treated Preparations

ber					
4		5		6	
D	C	D	C	D	C
28	31	11	24	23	17
32	26	22	29	9	15
25	21	23	37	13	15
44	46	18	28		
32.3±4.18	31.0±5.41	18.5±2.74	29.5±2.83	15.0±4.18	15.7±0.71
6.84		3.89		4.18	
0.190		2.736		0.164	
p >0.50		0.05 > p >0.02		p >0.50	

recordings of scanned bands. The areas are expressed as % of

e deficient and control histones.

les.

Comparison of Histone Disc

Series No.	No. of rats	1		2		3	
		D	C	D	C	D	
IX	4	8	10	10	17	12	
IX	6	11	6	14	19	13	
XI	10			24	28	17	
XIV	10	7	5	30	23	19	
Mean ± S.E.		8.7±1.23	7.0±1.53	19.5±4.56	21.8±2.45	15.3±1.66	1
S.E.		1.98		5.22		2.81	
t		0.857		0.441		0.28	
p		p > 0.10		p > 0.50		p > 0.	

For explanations refer to Table 3.

Table 4

lectrophoretic Bands in EIA Preparations

Band Number						
	4		5		6	
C	D	C	D	C	D	C
12	38	32	33	29		
13	35	34	26	21	3	7
20	35	35	24	18		
13	28	26	23	39		
.5±1.87	34.0±2.12	31.8±2.00	26.5±2.24	26.8±4.69		
	2.93		5.22			
	0.752		0.058			
0	p > 0.10		p > 0.50			

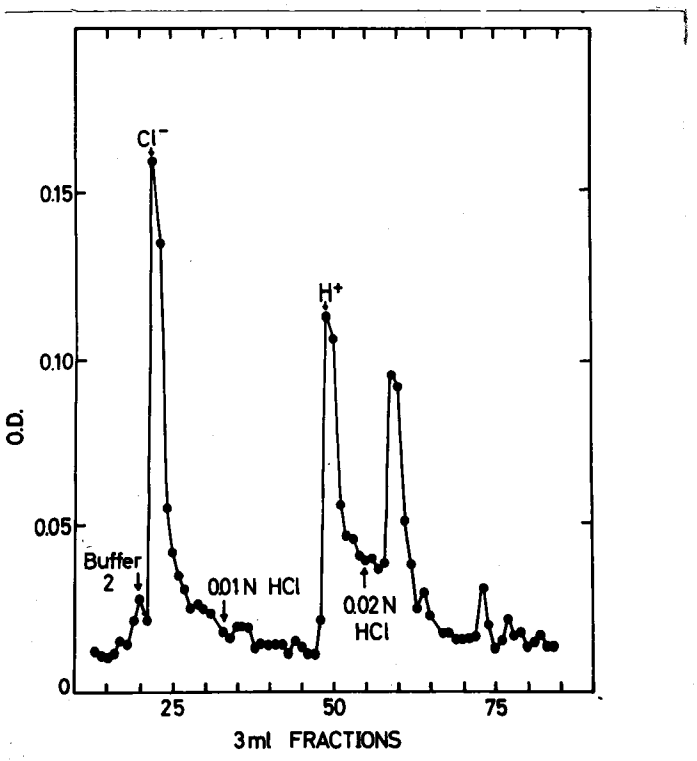

FIGURE 6. C.M. CELLULOSE CHROMATOGRAPHY OF HISTONES FROM ZINC DEFICIENT RATS.

The absorbance at 278 mμ was read.

10 mg of sample was applied to 20 x 1 columns.

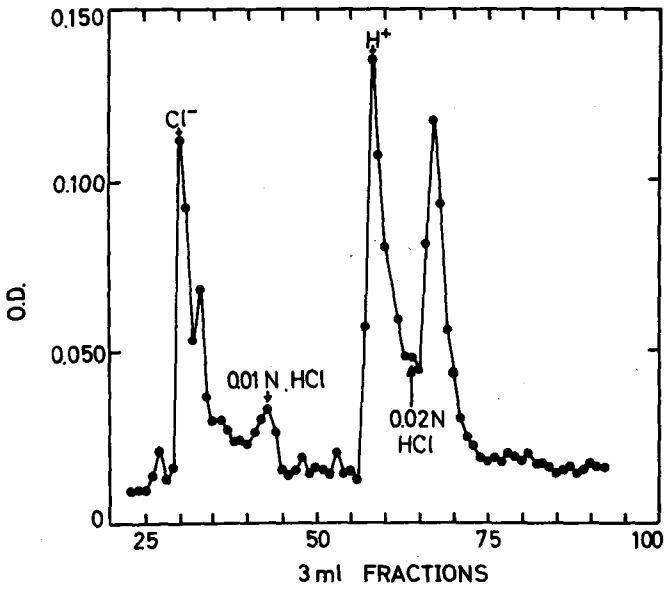

FIGURE 7. C.M. CELLULOSE CHROMATOGRAPHY OF HISTONES FROM CONTROL RATS.

The absorbance at 278 mμ was read.

10 mg of sample was applied to 20 x 1 columns.

retained by the adsorbent column indicating the presence of less basic components. The 278 mμ absorption peaks correlated with those at 750 mμ (Fig. 8), obtained by treatment with the Folin-Ciocalteu reagent (97).

The disc electrophoretic patterns of chromatographically separated fractions F_1, F_2 and F_3 from zinc deficient and control rats were identical (Fig. 4). The F_1 fraction contained 9 bands, F_2 and F_3 showed four bands each. Fast mobility bands appeared only in F_1. Fractions F_2 and F_3 showed the bands number 2 to 5. The F_2 differed from F_3 in the intense staining of zones 4 and 5 (Fig. 4).

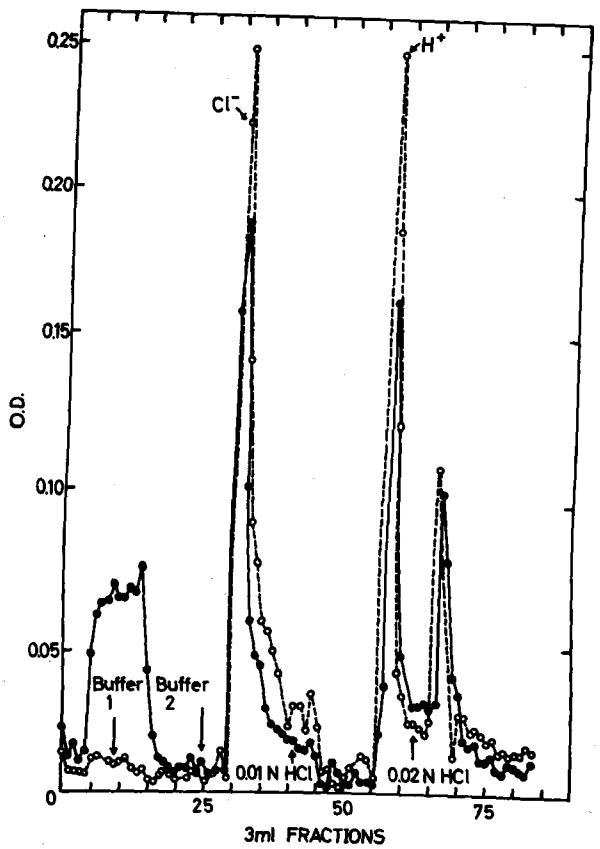

FIGURE 8. C.M. CELLULOSE CHROMATOGRAPHY OF RAT LIVER HISTONES.

●——●——●——● Absorbance at 278 mμ.

o——o——o——o Color developed by Folin-Ciocalteu reagent read at 750 mμ.

The scale on the right is in 0.100 units.

12 mg of histone was applied.

CHAPTER VI

DISCUSSION AND CONCLUSION

Histones are postulated to be genetic repressors. Evidence that histones are capable of inhibiting DNA replication (86) and of m-RNA synthesis (82, 83) supports such a hypothesis. Further support for a repressor function is provided by experiments in which it was shown that acetylation of histones prior to stimulation of m-RNA synthesis in activated lymphocytes eliminated the repressor action (69).

In zinc deficiency protein synthesis and growth are impaired. RNA levels are lowered (28), unlike DNA which appears to be unaffected (100). Inhibition of transcription of genetic information from DNA to RNA and subsequently to protein by a repressor might explain decreased RNA levels and lowered protein synthesis. Hence, the effect of zinc depletion on the behavior of histones is of considerable interest. Detection of changes of type or concentration of nuclear histones might explain in part the molecular basis of the effects of low levels of zinc and strengthen further the histone-repressor hypothesis.

Two fundamental requirements had to be fulfilled in order to obtain a successful treatment of the problem.

Severe zinc deficiency had to be induced in a suitable animal and histones had to be isolated in pure form. The rat was selected because it is susceptible to zinc deficiency. The liver as the major site of protein synthesis would be expected to show metabolic disturbances related to this function. Further, the liver contains substantial amounts of zinc. Therefore, a severe limitation on intake of zinc should result in the appearance of adverse effects in the liver caused by or related to zinc deficiency.

Zinc was almost completely removed from the food and precautions were taken so that no zinc other than provided in the diet was available to the rats. The impaired growth and various physical signs of deficiency gave evidence that severe zinc deficiency existed.

The preparation of histones from the nuclei was preferred because basic proteins are known to occur in the microsomes (101) and perhaps elsewhere. Proteolytic degradation was kept minimal by rapid isolation and purification methods carried out at low temperatures and by the use of protease inhibitors. The DNP histones used in earlier experiments were subject to contamination in addition to enzymatic hydrolysis during the longer period of isolation. Hence, DNP preparations were abandoned and nuclear histones used throughout the experiments.

The mode of preparation, solubility, electrophoretic behavior, and adsorption to and elution from C.M. Cellulose cation exchange columns shows that the isolated products were nuclear basic proteins. The differences in number, mobility and pattern of disc electrophoregrams of calf thymus and rat liver histones demonstrate that histones are a complex of closely related proteins. Although up to 12 bands were detected in the acrylamide gels, the number of individual histones may be smaller, some of the minor bands may represent decomposition products. Treatment of histones with urea or electrophoresis at lower pH produces more bands. A limited number of histones restricts repressor action to changes in histone type or amount. Hence levels of histones in the nucleus may be significant (33, 85).

Histones from zinc deficient and control rats compared electrophoretically showed a significant change in DFP treated preparations. However, EIA preparations failed to show such a difference. The demonstration of statistically significant differences despite the small number of assays indicates that histones undergo changes during zinc deficiency. The finding is compatible with a histone-repressor hypothesis. However, the possibility that disturbance of histone synthesis like that of other proteins occurs cannot be excluded.

The chromatographic behavior on C.M. Cellulose of histones from normal and deficient rats was identical. Resolution by means of other chromatographic media may be more successful and should be attempted. Immunoelectrophoretic studies seem worth doing.

In conclusion, histones from liver in zinc deficient rats undergoes a significant change detectable by disc electrophoresis. The cause for the discrepency between the DFP and EIA treated histone preparations remains to be established. It is possible that apart from a protease inhibiting effect these alkylating agents combine with histones chemically.

SUMMARY

1. Liver nuclear histones from zinc deficient and control rats were isolated and compared by electrophoresis on acrylamide gels and by chromatography on carboxymethyl cellulose.

2. Histone preparations in which diisopropylfluorophosphate (DFP) was used to inhibit proteolytic degradation differed from those in which ethyliodoacetate was used.

3. In the presence of DFP, histones isolated from liver nuclei of zinc deficient rats showed statistically a significant change in relative concentration of major histone fractions as compared with similar preparations from liver nuclei of control rats, when subjected to electrophoresis on acrylamide gels at pH 4.3.

4. Carboxymethyl cellulose chromatography failed to demonstrate differences between the deficient and control groups.

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